SALT TOLERANT L-MYO-INOSITOL 1-PHOSPHATE SYNTHASE AND THE PROCESS OF OBTAINING THE SAME

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] This invention relates to a salt tolerant L-myo-inositol 1-phosphate synthase and the process of obtaining the same.

Description of Related Art

[0002] In agricultural biotechnology a long standing goal is to improve tolerance of crop plants to environmental stress such as salinity, drought and temperature mediated dehydration all of which constitute direct osmotic stress. One of the mechanisms by which plants respond to such abiotic stress conditions is by synthesizing non-toxic biomolecules termed compatible solutes or osmoprotectants. These compounds fall into three categories; amino acids (eg proline), onium compounds (eg glycinebetaine, dimethylsulphoniopropionate) and polyols/sugars (eg inositol, ononitol/pinitol mannitol, trehalose). Over production of any such osmoprotectant by introgression of genes encoding critical steps in the synthesis of these compounds through metabolic engineering has become the choice of biotechnologists for raising stress tolerant crop plants. Such approaches have met limited success in both prokaryotic and eukaryotic systems. More importantly, it is imperative that the critical step for manipulation should itself encode a stress-tolerant enzyme protein.

[0003] Although metabolic engineering involving overproduction of selected osmolytes has been a choice for imparting stress tolerance phenotype in plants and other organisms, none of the systems used any stress tolerant gene/enzyme for such work. Hence, functional expression of the target gene/enzyme in the transgenic system remained unpredictable.

OBJECTS OF THE INVENTION

[0004] An object of this invention is to produce a salt-tolerant L-myo-inositol 1-phosphate synthase gene.

[0005] Another object of this invention is to provide a process for obtaining a salt tolerant genie for inositol production.

[0006] Yet another object of this invention is to introgress the salt tolerant L-myo-inositol 1-phosphate synthase in model crop plants for its functional expression to confer ability to grow in presence of salt without decline in photosynthetic functions.

BRIEF DESCRIPTION OF THE INVENTION

[0007] The present invention, provides a salt-tolerant L-myo-inositol 1-phosphate synthase from *Porteresia coarctata*.

[0008] Also provided in accordance with the present invention is a process of obtaining a salt tolerant myo-inositol 1 phosphate synthase gene comprising:

[0009] (i) isolation of a full-length cDNA for the L-myo-inositol 1-phosphate synthase gene from the leaf of Porteresia coarctata (PcINO1) by reverse transcription followed by polymerase chain reaction;

[0010] (ii) sequencing of the isolated L-myo-inositol 1-phosphate synthase gene;

[0011] (iii) Cloning of the isolated full length cDNA of PcINO1 in suitable bacterial expression vectors to obtain the expression plasmid construct.

[0012] Introduction of the expression plasmid construct into the bacterial host strain, E. coli BL21 (DE 3) by transformation and induction of expression of to PcINO1 gene project by IPTG.

[0013] Isolation of the expressed PcINO1 gene product as inclusion bodies solubilization and isolation of the active enzyme protein in a buffer containing 8M Urea, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM .beta.mercaptoethanol (ME) and 2 mM phenylmethylsulphonylfluoride (PMSF) and its complete purification to homogeneity.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] FIG. 1: (A) SDS-PAGE analysis of proteins of bacterially expressed RINO1 and PcINO1; lanes 1&2-RINO1, induced and uninduced; lanes 3 & 4- PcINO1 induced and uninduced; lanes 5 & 6-control-induced and uniduced. (1) Corresponding western blot of (A).

[0015] FIG. 2: (A) SDS-PAGE of proteins in pellet and supernatant fraction in the induced system after urea solubilization; lanes 1 and 2, pellet and supernatant of induced RINO1; lanes 3 & 4-pellet and supernatant of induced PcINO1. (B) corresponding western blot of (A).

[0016] FIG. 3: Inositol synthase activity in presence of increasing NaCl concentration for purified native (A) and recombinant (B) enzymes.

[0017] FIG. 4: Tryptophan fluorescence of purified RINO1 (A) and PcINO1 (B) proteins in increasing NaCl concentrations; tracing 1,2,3 & 4 correspond to 0, 100 mM, 20 mM and 400 mM NaCl in the system.

[0018] FIG. 5: Gel filtration pattern on Superose-12 of RINO1 and PcINO1 proteins in absence and presence of 400 mM NaCl. (A) RINO1 without NaCl; (B) RINO1 with NaCl; (C) PcINO1 without NaCl; (D) PcINO1 with NaCl. Insets depict SDS-PAGE and immuno dot blots of indicated fractions.

[0019] FIG. 6: Circular Dichroism spectra of RINO1 and PcINO1 proteins.

[0020] FIG. 7: Phenotype of nontransformed and PcINO1-transformed tobacco plantlets grown with various concentration of NaCl in the growth media.

DETAILED DESCRIPTION OF THE INVENTION

[0021] Cloning and sequencing of L-myo-inositol 1-phosphate synthase ~~gene~~ from *Porteresia coarctata* (PcINO1) and its comparison with that from *Oryza saliva* (RINO1).

[0022] A full length cDNA for the L-myo-inositol 1-phosphate synthase gene has been obtained from Porteresia coarctata (PcINO1) as well as Oryza sativa (RINO1) leaf poly-A (RNA) by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from mature leaves of Oryza and Porteresia following the method of Ostrem et al (Plant Physiol. 84, 1270-1275,1987). Poly-A RNA was isolated from the total RNA by the polyAtract mRNA isolation kit (Promega) following the manufacturer's instructions. 20-30 ng of poly-A RNA was used for first stand cDNA synthesis using Superscript II RNAse H-reverse transcriptase (Life Technologists; Gibco BRL) following the manufacturer's protocol. cDNA thus synthesized was used as template for PCR amplification of the inositol synthase gene. For cloning of the full-length cDNA of inositol synthase for Oryza (RINO1) and Porteresia (PcINO1), sense (5'-3') and anti-sense (3'-5') oligonucleotide primers were designed based on the published RINO1 sequences (GenBank accession number AB 012107) and PCR amplification was done as follows: 94-1 min[94-1.5 min; 55, 1.5 min; 72, 2 min].times.32 cycles; 72, 10 mins. The amplified product was checked for the expected size (.about.1.5 kb), band eluted from the gel, purified through QIAquick PCR purification kit (Qiagen) and ligated overnight at 4°C. to the pGEM T-Easy vector (Promega) following manufacturer's instructions. The ligation mixture was used for transformation of high efficiency JM109 competent cells (Promega) and transformants were selected based on blue/white selection on ampicilin/IPTG/X-gal plates grown overnight Minipreps of the plasmids were isolated from the transformants, the DNA digested with EcoR1 and the digested DNA analyzed by agarose gel electrophoresis for the expected .about.1.5 kb insert. Having confirmed the insert size, plasmid DNA was isolated from the transformants and

purified through the Qiaquick purification it (Qiagen). The clones were designated as RINO1 for the gene for inositol synthase from Oryza sativa and PcINO1 for the same from Porteresia coarctata.

[0023] The nucleotide sequence for each clone was determined through automated DNA sequencing. The sequencing strategy involved several cycles of sequencing of the clones by designated primers as follows:

[0024] 1. First round with primers for T_7 promoter at the 5' end and SP.sub.6 promoter at the 3' end.

[0025] 2. Second round with primers designed at the 5' end and the 3' end of the cDNA as used for RT-PCR amplification.

[0026] 3. Third round of sequencing with primers designed at about 250 base pairs downstream the start site and 250 nucleotides upstream of the stop site

[0027] The sequencing data from each set were compiled and compared to work out the complete sequence of the L-myo-inositol 1-phosphate synthase from Porteresia coarctata (PcINO1) and Oryza sativa (RINO1, GenBank accession number AB012107). The complete sequence of PcINO1 is provided hereunder:

[0028] PcINO1 DNA Sequence (SEQ. ID 1) and PcINO1 amino acid Sequence (SEQ. ID 3):

atg ttc atc gag agc ttc cgc gtg gag agc ccg cac gtg cgg tac ggc gcg gcg gag atc

M F I E S F R V E S P H V R Y G A A E I

gag tcg gag tac cgg tac gac act acg gag ctg gtg cac gag agc cac gac ggc gcc tcg

E S W Y R Y D T T E L V H E S H D G A S

cgc tgg gtc gtc cgc ccc aag tcc gtc cag tac cac ttc agg acc agc acc acc gtc ccc

R H V V R P K S V Q Y H F R T S T T V P

aag ctc ggg gtc atg ctc gtg ggg tgg ggc ggc aac aac ggc tca acg ctg acg gct ggg

K L G V M L V G W G G N H G S T L T A G

gtc atc gcc agc agg gag gga atc tca tgg gcg acc aag gac aag gtg cag caa gcc aac

V I A S R E G I S W A T K D K V Q Q A N

tac tat ggc tca ctc acc cag gcg tcc acc atc agg gta gga agc tac aac ggg gag gag

Y Y G S L T Q A S T I R V G S Y N G E E

atc tac gcg cct ttc aag agc ctc ctg ccc atg gtg aac cct gat gac ctt gtg ttc ggg

I Y A P F K S L L P M V N P D D L V F G

ggc tgg gac att agc aac atg aac ctg gct gat gct atg acc agg gcc aag gtg ctg gac

G W D I S N M N L A D A M T R A K V L D

att gat ctg cag aag cag ctt agg cct tac atg gag tcc tgg tgc ctc tcc ctg gca tct

I D L Q K Q L R P Y M E S W C L A L A S

atg atc ccg act tca tcg ccg cta acc agg gat ccc gcg cga aca atg tca tca agg gaa

M I P T S S P L T R D P A R T M S S R E

cca aga agg agc aga tgg ggc aga tca tca aag gac atc agg gag ttc aag gaa aat aac

P R R S R W G R S S K D I R E F K E N N

aaa atg gac aag gcg gtg gtg ttg tgg act gca aac act gaa agg tac aac aat tgt ctg

K M D K A V V L N T A N T E R Y N N C L

tgt ttg ggc tta atg acc aat gga aaa cct tct gcg tct gtg gac agg aac cag gcg gag

C L G L M T N G K P S A S V D R S Q A E

ata tcg cca tcg aca ttg tat tgc cat tgc ctt gct tca ttg gag ggt gtc cgt tca ata

I S P S T L Y C H C L A S L E G V R S I

acg gga gcc ctt aaa aaa aaa tct tgg cct gga att gac gat ctt gcc att aaa aaa aaa

T G A L K K K S W P G I D D L A I K K K

ctg cct gat ccg ggg gga tta att caa aaa agg ggc aaa cca aaa aaa aaa acc ggc ttg

L P D P G G L I Q K R G K P K K K T G L

gtt gat ttc ctc atg ggt gct gga ata aag ccc acc tca att gtc agt tac aac cac ttg

V D F L M G A G I K P T S I V S Y N H L

ggg aat aat gat ggc acg aac ctt tct gcg ccg caa aca ttc cga tcc aag gag atc tcc

G N N D G T N L S A P Q T F R S K E I S

aaa agc agc gtg gtc gat gac atg gtc tca agc aat gct atc ctc tac gag cct ggc gag

K S S V V D D M V S S N A I L Y E P G E

cat cct gat cat gtt gtc gtg att aag tat gtg ccg tac gtc gga gac agc aag agg gcc

H P D H V V V I K Y V O Y V G D S K R A

atg gat gag tac acc tca gag atc ttc atg ggg ggt aag aac acc atc gtg ctg cac aac

M D E Y T S E I F M G G K M T I V L H N

acc tgc gag gac tcg ctc ctt gct gca cca atc att ctt gac ctg gtg ctc ctg gcc gag

T C E D S L L A A P I I L D L V L L A E

ctc agc act agg att cag ctg aaa ggc gag gga gag gag aaa ttc cat tcc ttc cat cca

L S T R I Q L K G E G E E K F H S F H P

gtg gct acc atc ctg agc tac ctc acc aag gcg ccc ctt gtt cct cct ggc aca cca gtg

V A T I L S Y L T K A P L V P P G T P V

gtg aac gcc ctg gcg aag cag agg gct atg ctc gag aac atc atg agg gcc tgc gtt ggg

V N A L A K Q R A M L E N I M R A C V G

ctg gcc cct gag aac aac atg atc ctg gag tac aag

L A P E N N M I L E Y K

[0029] The sequence has also been submitted to the GenBank (Accession Number AF 412340) and will be held confidential until Jun. 23, 2003.

[0030] Comparison of the amino acid sequence of PcINO1 (SEQ ID 3) with that of RINO1 (SEQ ID 2) is illustrated in FIG. 1.

[0031] On analysis, and as seen in FIG. 1, it was revealed that the nucleotide sequences of PcINO1 (SEQ ID 1 and SEQ ID 3) is considerably non-identical resulting in products in which the RINO1(SEQ ID 2) and PcINO1 (SEQ ID 1 and SEQ ID 3) differ in the amino acid sequences for a stretch of about 110 in the mid-portion (between amino acids 173 to amino acids 320 of PcINO1 (SEQ ID 3), the other parts of the sequences bearing complete identity. The non-identical portion is comprised of deletions/additions as well as conservative substitutions with two additional amino acids in case of PcINO1 resulting in a protein having 512 amino acids instead of reported 510 amino acids of RINO1.

[0032] Expression of RINO1 and PcINO1 in Bacterial Expression Vectors:

[0033] The cDNA for RINO1 and PcINO1were subcloned into suitable cloning sites of the bacterial expression vector pET 20B (+). The resulting plasmids were introduced into the host strain E. coli BL-21 (DE3). The bacteria were grown in LB medium up to A_{600} of 0.5-absorbance unit and induced by 0.5 mM IPTG for 6 hours at 30°C. The bacteria were collected by centrifugation and lysed by sonication in a buffer containing 20mM Tris-HCl, pH 7.5, 10 mM each of NH_4Cl and ME, and 2mM PMSF. The lysed extracts were centrifuged and protein from both soluble and membrane fractions were analyzed by 10% SDS-PAGE according to Laemmli (Nature, 227, 680-685, 1970) followed by western blot for immunodetection. The separated proteins were blotted onto PVDF membrane and the blot was probed with rabbit anti L-myo-inositol 1-phosphate synthase antibody (1:500) raised against purified recombinant L-myo-inositol 1-phosphate synthase of *Entamoeba* (Lohia et al, Mol.Biochem.Parasitol., 98, 67-79,1999) or purified cytosolic L-myo-inositol 1-phosphate synthase from Oryza leaves. Bound antibody was detected by the chemiluminiscence (kit from Amersham Life Sciences). Results of such experiments indicated that both RINO1 and PcINO1 were expressed predominantly in the membrane fractions (Fig 2, A & B, lanes 1 & 3).

[0034] Solubilization of Expressed RINO1 and PcINO1 Proteins:

[0035] The expressed RINO1 and PcINO1 proteins were solubilized from the pellet fractions in solubilization buffer (8M urea, 0.5 M NaCl, 20 mM Tris-HCl pH 7.5, 10 mM ME, 2 mM PMSF) kept for 30 minutes at room temperature. Solubilized samples were centrifuged at 15000 rpm for 30 minutes. Supernatant was taken and dialyzed serially in the same buffer with stepwise dilution of urea concentration from 8M to 2M. The solubilized samples were checked in SDS-PAGE and western blot for the

[0036] RINO1 and PcINO1 proteins (FIG. 3, A & B, lanes 2 & 4). After solubilization, SDA-PAGE analysis revealed that the expressed protein was in soluble fraction (FIG. 3A) and was again confirmed by western blot analysis (FIG. 3 B).

Purification of the Solublized RINO1 and PcINO1 Proteins:

[0037] The protein in the dialyzed sample was purified by DEAE Sephacel and Biogel A 0.5 by procedures earlier described from this laboratory (RayChaudhury et al., Plant Physiol., 115, 727-736,1997). Solubilized dialyzed sample was taken and loaded onto DEAE Sephacel column (20 ml bed volume). After two hours of absorption of the protein onto the column, the effluent was collected and then washed in buffer A containing 20 mM Tris-HCl, pH 7.5, 10 mM each of NH_4Cl and ME, 2 mM PMSF, 20% glycerol upto nearly 3 bed volume for elution of unbound protein and until the A.sub.280 of the fractions approached 0. Bound proteins were eluted in 60 ml linear gradient of 0.01 to 0.25M NH_4Cl in buffer A. Fractions of 1 ml were collected at the rate of 0.4 ml/min. Fractions with inositol synthase activity were pooled, concentrated and dialyzed for 6 hr at 4°C. against 2 L change of buffer A. The dialyzed and concentrated, pooled DEAE fractions were loaded on a Biogel A 0.5 column, preequilibriated with 3 bed volumes of buffer A. Proteins were eluted with buffer A in fractions of 0.5 ml at a flow rate of 0.1 ml/min. Fractions containing inositol synthase activity were pooled, dialyzed against one 2 L change of 20 mM Tris-Cl (pH 7.5) 10 mM ME.

Biochemical Characterization of the Expressed RINO1 and PcINO1 Proteins

[0038] The purified bacterially expressed RINO1 and PcINO1 proteins were characterized for their biochemical properties (Table-1). Estimates of Km and Vmax values for the substrate (Glucose 6 phosphate) and co factor (NAD) were obtained with Biogel 0.5A purified recombinant synthase (s) using Line-Weaver Burk analysis. There is a difference between the Km values for glucose 6 phosphate of recombinant synthase of Oryza (RINO1) and Porteresia (PcINO1). The lower Km values for glucose 6 phosphate for recombinant synthase of Porteresia (PcINO1) suggest a higher substrate specificity compared to the Oryza

recombinant synthase (RINO1). For both the cases optimum enzyme activity was at 37° C. whereas optimum pH for Porteresia recombinant synthase (PcINO1) was 8.0 and the same for Oryza recombinant synthase (RINO1) was 7.5.

[0039] With respect to salt-sensitivity, RINO1 and PcINO1 proteins differ a great deal. As in the case of the purified native enzymes (FIG. 4,A), the expressed recombinant RINO1 and PcINO1 proteins exhibit similar characteristics with respect to salt-sensitivity/tolerance properties (FIG. 4, B). It is evident that both native and recombinant RINO1 proteins are sensitive to NaCl in vitro, whereas those of PcINO1 are tolerant to salt under if vitra conditions upto a concentration of 500 mM NaCl adducing evidence that the expressed gene products of both retain their salt-sensitivity vis-a-vis salt-tolerance properties like the native enzyme proteins.

TABLE 1

Biochemical characterization of native and recombinant RINO1 and PcINO1 proteins				
	PINO		RINO	
Characters	Native	Recombinant	Native[+]	Recombinant
Km				
(1) G6P	1.81 mM	2.5 mM	1.97 mM	3 mM
(2) NAD	0.153 mM	0.166 mM	0.14 mM	0.188 mM
Vmax				
(1) G6P	0.08 μmol^{-m}	0.095 μmol^{m}.	0.07 μmol^{-m}	0.072 μmol^{-m}
(2) NAD	0.12 μmol^{-m}	0.087 μmol^{m}	0.09 μmol^{-m}	0.068 μmol^{-m}
pH optimum	7.5	8.0	8.2	7.5
Temperature	35°C	37 °C	35 °C	37 °C
Optimum Molecular Weight				
Native	~180 kDa	~180 kDa	~180 kDa	~180 kDa
Subunit	~60 kDa	~60 kDa	~60 kDa	~60 kDa

[+]Data from RayChaudhury et al. (1997)

[0040] The invention is described in greater detail hereinafter, with reference to the accompanying drawings and examples, which are provided as mere illustrations of the invention and should not be construed to limit the scope thereof in any manner.

EXAMPLE

Structural Studies of RINO1 and PcINO1: Fluorescence, Circular Dichroism and Gel-Filtration Studies

[0041] In order to understand the structural basis of the differential behaviour of RINO1 and PcINO1 towards salinity stress, we performed some fluorescence, Circular Dichroism (CD) and gel filtration experiments.

[0042] Tryptophan Fluorescence spectra of the recombinant preparations of the inositol synthase(s) from Oryza sativa (RINO1) and Porteresia coarctata (PcINO1) are shown in Panel A and B respectively in FIG. 4. In absence of added salt, RINO1 shows significantly higher fluorescence intensity than PcINO1 at the wavelength of maximum emission. The emission maxima in both the cases remain close to 336 nm. Fluorescence intensity of RINO1 is quenched significantly in presence of added salt whereas that of PcINO1 is rather insensitive. It is also interesting to note that at salt concentration of over 600 mM, the fluorescence intensities of both RINO1 and PcINO1 become comparable.

[0043] Progressive decrease of fluorescence intensity of RINO1 with increasing salt concentration indicates structural alterations. However, the emission maximum of RINO1 remains invariant as a function of increasing salt concentration meaning that the tryptophan environment remains unchanged. Tryptophan residues usually remain buried within the globular structure. Therefore the salt-induced changes do not interrupt the tryptophan microenvironment. It probably moves other protein segments closer to tryptophan to facilitate energy transfer and hence reduce intensity. The structure of PcINO1 is stable to addition of salts. Since salts screen electrostatic interaction, there is considerable difference in the exposition of charged residues on the outer surface of RINO1 and PcINO1.

[0044] The structure of RINO1 and PcINO1 proteins in solution at the secondary level was probed by the Far-UV Circular Dichroism(CD) spectroscopy. The CD spectra of RINO1 and PcINO1 proteins are almost identical in shape and show the characteristic bands (FIG. 5). The spectra were subjected to three-parameter secondary structure analysis Helix, sheet and random) by a non-linear curve fitting analysis according to K2D programme available from the K2D server on the Internet The analysis reveals that both RINO1 and PcINO1 have very similar secondary structural elements having -25% α-helix, -25% β-sheet and 50% random plus other structures. Clearly the differences in the two proteins arise because of the different ways these secondary structural elements pack together.

[0045] In order to get ether insight into the nature of the structural changes in RINO1 due to addition of salt, we performed gel-permeation chromatography of RINO1 and PcINO1 proteins both m presence and absence of added salt, the chromatograms are shown in FIG. 6. (A,B,C & D). It is seen that while RINO1 protein elutes as a single peak in absence of salt,

addition of 400 mM NaCl leads to substantial reduction in the original peak and concurrent appearance of a high molecular weight fraction, suggesting oligomerization of the RINO1 protein by addition of NaCl. In contrast, PcINO1 protein elutes at the same place corresponding the native trimeric association, both in absence and presence of NaCl. The activity data show that the trimeric form of the protein is enzymatically active although the oligomeric form (of the size of a tetramer) is inactive. Since oligomerization would affect mainly the protein surface and not the globular interior, the salt-sensitivity of the RINO1 protein may be explained by a suggested mechanism involving difference in ionic environments prevailing on the surface and a difference in hydrophobicity close to the surface when compared to the salt-tolerant PcINO1 protein.

Phenotype of Tobacco Plants Transformed with PcINO1 Gene During Salt-Growth

[0046] To determine whether introgression of PcINO1 into plant system may help growing the plant in presence of salt, tobacco plants transformed with the PcINO1 gene through the Agrobacterium-mediated procedure were raised. For this, the PcINO1gene was cloned into the plant expression vector, pCAMBIA 1301 and mobilized into tide Agrobacterium strain LBA 4404 by following standard procedures. Tobacco leaf discs, precultured in regeneration media were immersed in the suspension of Agrobacterium culture containing the PcINO1-pCAMBIA construct for 1 hr and transferred back to the regeneration medium supplemented with cefotaxim and hygromycin. After shoot and root growth, the regenerated plantlets were transferred to culture vessels containing 0, 100, 200 and 400 mM NaCl for further growth. Control plantlets transformed with only pCAMBIA vectors were also grown in salts in similar way. A comparison of the plants (control and the PcINO1-transformed) grown in presence of increasing amount of NaCl show that while the control plants exhibit loss of chlorophyll in presence of 200 mM NaCl, the PcINO1 transformed plants exhibit no such loss of chlorophyll at the indicated salt concentration, although at 400 mM salt both types of plants fail to grow (FIG. 7). This might suggest that the PcINO1 transformed plants are able to maintain the photosynthetic machinery in presence of NaCl at concentrations normally inhibitory to the growth of untransformed plants.

[0047] The above-mentioned experiments strongly 'suggest that the PcINO1gene sequence(s) may become a useful tool for production of transgenic crop plants tolerant to salt stress.

[0048] While the invention has been described in details and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and

modifications can be made therein without deviating or departing from the spirit and scope of the invention Thus the disclosure contained herein includes within its ambit the obvious equivalents and substitutions as well.

[0049] Having described the invention in detail with particular reference to the illustrative examples and comparative data given above, it will now be more specifically defined by means of claims appended hereafter.